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14. ABSTRACT Polytrauma is most often caused from explosive devices and accounts for about 65 percent of injuries to our military personnel. The patients who have polytrauma are at increased risk of developing either bleeding and/or a clot in their veins which cause a life-threatening event known as venous thromboembolism (VTE). We began enrollment of patients into the study on 2 February 2011. As of 1 October 2012, we have successfully enrolled and collected blood samples on 684 patients and 64 healthy volunteers. We have thus far analyzed plasma samples of over 230 patients and 64 volunteers. In our preliminary analysis of thrombin generation and procoagulant microvesicle analysis, we have observed that thrombin generation is accelerated early after traumatic injury and there are greater numbers of procoagulant microvesicles noted after traumatic injury relative to healthy volunteers.

15. SUBJECT TERMS
Polytrauma, thrombin generation, venous thromboembolism

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Introduction: Venous thromboembolism (VTE) is a combat casualty adverse event reported to the Department of Defense. Rates of symptomatic and asymptomatic deep vein thrombosis (DVT) and pulmonary embolism (PE) in trauma patients are as high as 44 and 24%, respectively. The current guideline is that all major trauma patients receive VTE chemoprophylaxis. This practice exposes those not at risk for thrombosis to potentially serious bleeding and there are no adequate laboratory tests currently available to target anticoagulant prophylaxis to those that need it most. The **central hypothesis** of this proposal is that traumatic injury results in the release of procoagulant and pro-inflammatory factors found both in plasma and microvesicles (MVs) derived from blood cells and injured tissues. The specific Aims of this study are:

Aim 1:
- Identify cellular origins and quantitate procoagulant microvesicles (MVs) defined by cell specific markers in patients with acute traumatic injury.
- Determine the basis of differences in thrombin generation via Calibrated Automated Thrombogram (CAT).

Aim 1 will be achieved through a prospective cohort study of patients with major trauma, estimate the distribution over time of procoagulant MVs concentration by cell of origin and thrombin generation.

Aim 2: Develop a predictive signature for a pre-thrombotic individual: thrombin generation concurrent with thrombogenic microvesicles.

Key Words:

Trauma, Venous Thrombembolism (VTE), Microvesicles, Thrombin

Research Accomplishments:
The major goal of the project was to assess if traumatic injury results in the release of procoagulant and pro-inflammatory factors found both in plasma and microvesicles (MVs) derived from blood cells and injured tissues. Furthermore, our goal was to develop a predictive signature for a pre-thrombotic individual: thrombin generation concurrent with thrombogenic microvesicles i.e., to assess whether individuals with elevated thrombin and microvesicles are at increased risk for VTE
- Standardization of methods to perform the MV analysis by flow cytometry and thrombin generation by calibrated automated thrombinogram (CAT).
- When performing CAT, reference plasma should be used in conjuction with patient samples to assess for any lot-to-lot variability of reagents purchased commercially. (Appendix: Figure 1)
- Screened 2234 trauma patients and enrolled 1139 patients and 89 volunteers into the proposed study.
- Plasma sample analysis of 443 patients performed each patient having an average of three samples for CAT and MV analysis.
- Among 443 trauma patients (1734 samples; ISS=13.0 [6.0, 22.0], hospital LOS=4.0 [2.0, 10.0] days, age=48 [28, 65] years, 70.7% male, 95% with blunt mechanism, mortality 3.2%),

no discernable patterns in thrombin generation or MV concentration were observed over time. The peak height and MVs were significantly different from healthy volunteers and were 337 [285, 395] nM and 400 [211, 772] per uL plasma, respectively.

- Extreme (defined as highest or lowest 5%) values reflecting a possible "hypercoagulable state" (lagtime ≤ 1.98, peak height ≥ 486.2, ttPeak ≤ 3.61, and total procoagulant MV ≥ 2278) were reached within 12 hours after acute trauma, while extreme values representing a possible "hypocoagulable state" (lagtime ≥ 18.6, peak height ≤ 17.8 and ttPeak ≥ 29.45) were not reached until 1-3 days.

- We subsequently performed further analyses to assess if MV and thrombin generation kinetic data can be used as predictors for VTE. Although MV counts did not correlate with VTE, thrombin genetation parameter, ttPeak via CAT assay was significant predictor of symptomatic VTE after injury.

- Eight three patients developed VTE within 92 days after trauma; 11 patients developed VTE within day 1 and 35 patients (42%) developed VTE after hospital discharge.

- Of the 83 VTE cases, 11 patients developed VTE at end of day 1 and were considered to have non-preventable VTE. Hence, the following analyses were based on the remaining 72 VTE cases. In a univariable analysis of the prospective cohort data that included clinical characteristics available within 24 hours of admission (i.e., characteristics that would be available to assess the individual risk of VTE shortly after admission), several clinical variables, worst (shortest) ttPeak and Lagtime within first 24 hours of injury (if patients had multiple samples over time) were independent predictors of incident VTE within three months after acute trauma.

- For our multivariable model , we chose clinical variables that were significantly and strongly associated with VTE and easy to obtain within 24 hours after admission. This model showed that increasing patient age (1.30 [1.13,1.48], P=0.0002), body mass index (BMI) ≥ 30 kg/m2 (3.66[1.75,7.65], P=0.0005), any surgery that required general anesthesia (2.50[1.50, 4.16], P=0.0004) and most hypercoagulable (shortest) ttPeak value reached during initial 24 hours (1.67[1.29, 2.15], p <0.0001) were independent predictors of incident (first-lifetime time) VTE within 92 days after trauma (Table 1):

Table 1: Final Multiple Variable Cox Proportional Hazard Model - 24-Hour Independent Predictors of VTE after Trauma[#]

Characteristic	HR	95% CI	Beta Coefficient	Z-Statistic	p-value
25 ≤ BMI < 30	1.86	(0.86, 4.04)	0.622	1.57	0.116
BMI ≥ 30	3.66	(1.75, 7.65)	1.298	3.46	0.0005
Age, per 10 years	1.30	(1.13, 1.48)	0.259	3.77	0.0002
Any Surgery	2.50	(1.50, 4.16)	0.917	3.55	0.0004
Worst (shortest) ttPeak*	1.67	(1.29, 2.15)	0.510	-3.96	<0.0001

[#]Concordance Statistic of clinical variables is 0.728 (Increases to 0.759 with addition of ttPeak)
*Using reagents 5 pM TF/4 uM PS and *HR is per 1-minute decrease in ttPeak

- In a receiver operator characteristic curve analysis, a VTE risk score cutoff of 0.570 gave the best sensitivity and specificity of 74% and 74%, respectively. Hence, a patient at end of day 1 of admission who has a VTE risk score of 0.570 or greater would be considered to be at "High-Risk" for VTE in the ensuing 92 days. This definition led to 52 true positives, 18 false negatives, 78 false positives and 223 true negatives. The addition of ttPeak more accurately predicted VTE than using clinical variables alone (in our case, BMI, Age, Any Surgery). Specifically, we were able to identify (net) 7 more true VTE cases and 7 more true VTE negative patients, reducing the rate of misclassification by 13% (14/110) due to ttPeak datum in the model (Table2):

Table 2: Reclassification of Cases and Cohort Patients with addition of ttPeak to the Multivariable Clinical Model:

	True VTE (Yes)	True VTE (No)
Low to High-Risk	8	9
High to Low-Risk	1	16

- In conclusion, the individual's plasma coagulome (as reflected by thrombin generation) is an independent predictor of VTE after trauma. Clinical characteristics and ttPeak can be used to stratify acute trauma pts into high and low risk for VTE.

Impact:

The overall incidence of VTE remained relatively unchanged at about 1 per 1000 between 1980 and 2000, and actually increased during 2001-2005.[1-4] Likewise, after trauma, the incidence of pulmonary embolism has more than doubled during the period of 2007-2009 as compared to an earlier period (1994-2001). While the Joint Commission, CMS and the Surgeon General recommend assessment of the risk of VTE for every hospitalized patient, our poor understanding of mechanisms contributing to VTE has precluded development of reliable biomarkers and standardized screening procedures to identify the individual at high risk for VTE. Previously identified clinical risk factors for VTE after trauma have a low predictive value for the individual patient; fatal VTE may occur despite a presumed low risk profile. Hence, there is critical need for identifying the high VTE-risk individual trauma patient to decrease the risk of bleeding complications[6] or infection from the use of anticoagulant-based prophylaxis in the low VTE-risk individual.

We successfully enrolled and analyzed plasma samples of trauma patients. We estimated the distribution over time of procoagulant MVs by cell origin and thrombin generation (Aim 1) and found that, overall, patients are in a hypercoagulable state relative to their time of discharge. As evident on the trend plots over time, no overall specific pattern of CAT variables and MV counts were observed during the first 24 hours after injury. However, for those patients who develop "Extreme" CAT variables do so early after injury and they have consistently greater injury severity scores (ISS). Furthermore, in a multivariate Cox model that included clinical and CAT

characteristics available within 24 hours of admission, shortest ttPeak (1.67[1.29, 2.15], p <0.00001) was an independent predictors of incident VTE within 92 days after trauma.

This is the first study to show that an individual's plasma coagulome as reflected by thrombin generation (CAT) can be used to predict VTE after trauma. Current guidelines recommend VTE prophylaxis for all trauma patients, a patient population at high risk for bleeding. The literature is replete with well-designed work elucidating risk factors for VTE in trauma patients. However, follow-up often is limited to the duration of hospitalization. In our prospective case-cohort study, we observed that 42% of cases developed VTE after hospital discharge. This observation is very similar to findings by Godat et al., showing that the period of VTE risk after acute injury persists for 3 months after injury. Additionally, the peak endogenous thrombin generation formed more quickly as compared to non-VTE patients very early after injury. In particular, ttPeak value appears to be an independent predictor of VTE and helped us to identify (net) 7 more true VTE cases and 7 more true VTE negative patients; reducing the rate of misclassification by 13%. Hence, acceleration of thrombin generation is important in generating the hypercoagulable milieu needed for thrombotic complications. In our multivariable model, Z-scores for the beta-coefficients were on the order of 3 to 4, indicating high power to detect them again in a new sample of similar size. Currently, no appropriately validated tools, that takes into account individual patient's coagulation phenotype, are available to identify the individual trauma patient at high risk for VTE. Our multivariable model shows that ttPeak improves the concordance statistic from 0.727 to 0.758.

Changes/ Problems:
- Nothing to Report

Products:
- Due to the funding of the grant, we have analyzed MV data and recently published our findings on the effect of transfusion on the levels of procoagulant MVs: Dhillon SK, Houck ML, Jenkins DH, Rosedahl JK, Harmsen WS, Halling TM, Park MS. Transfusion of stored red blood cells in trauma patients is not associated with increased procoagulant microparticles. Accepted in J Acute Care Surg. 2014; 77(5): 674-678.
- Due to the funding of the grant, we have recently published our findings to identify potential mechanisms for venous thromboembolism and bleeding after acute trauma. we estimated changes in circulating procoagulant microvesicles and thrombin activity during hospitalization for trauma.Park MS, Xue A, Spears GM, Halling TM, Ferrara MJ, Kuntz MM, Dhillon SK, Jenkins DH, Harmsen WS, Ballman KV, Harrison P, Heit JA. Thrombin generation and procoagulant microparticle profiles after acute trauma: a prospective cohort study. J Trauma Acute Care Surg 2015;79(5):726-31.

Participants &Other Collaborating Oraganization:
No change

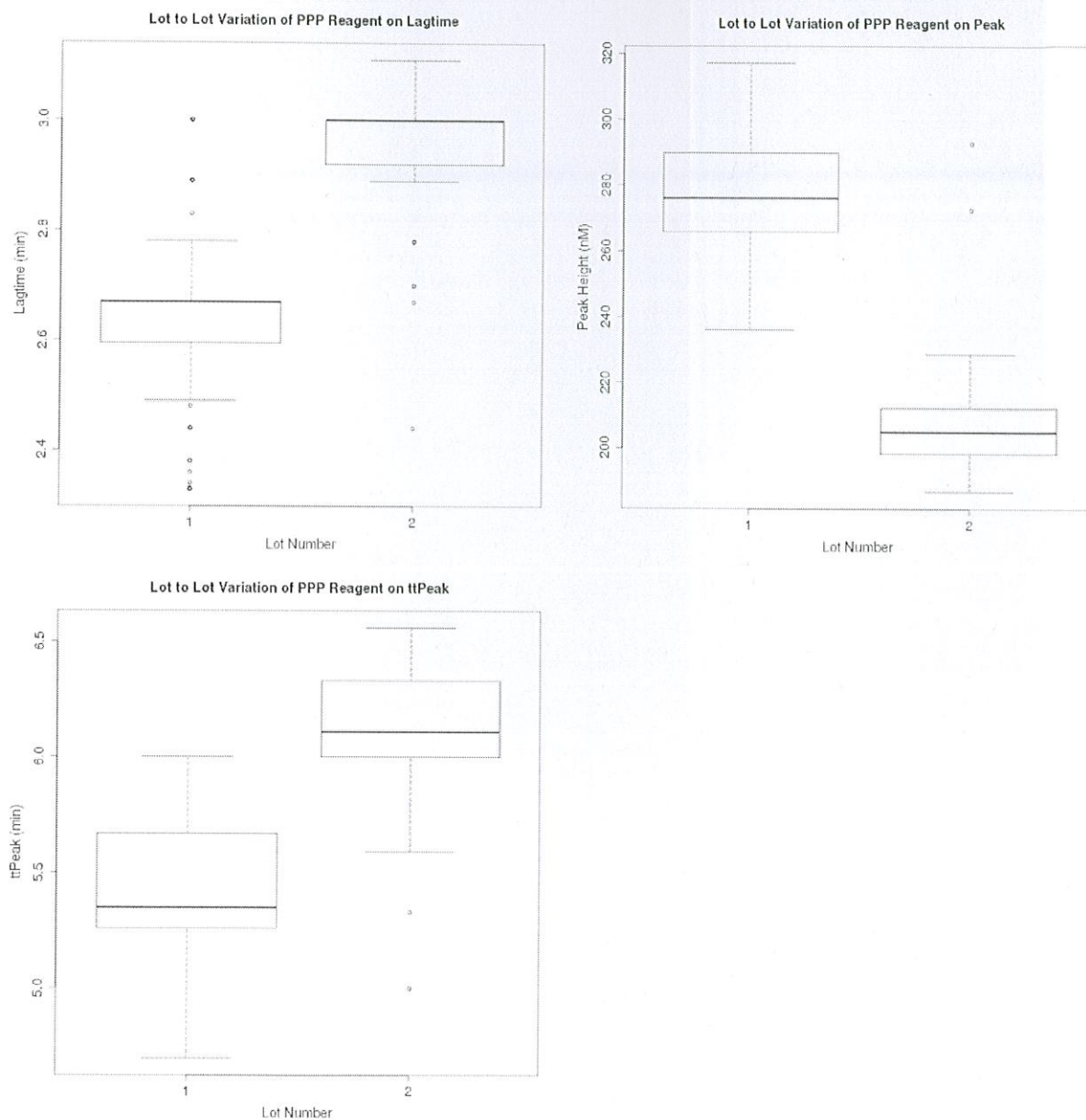## Special Reporting Requirements:
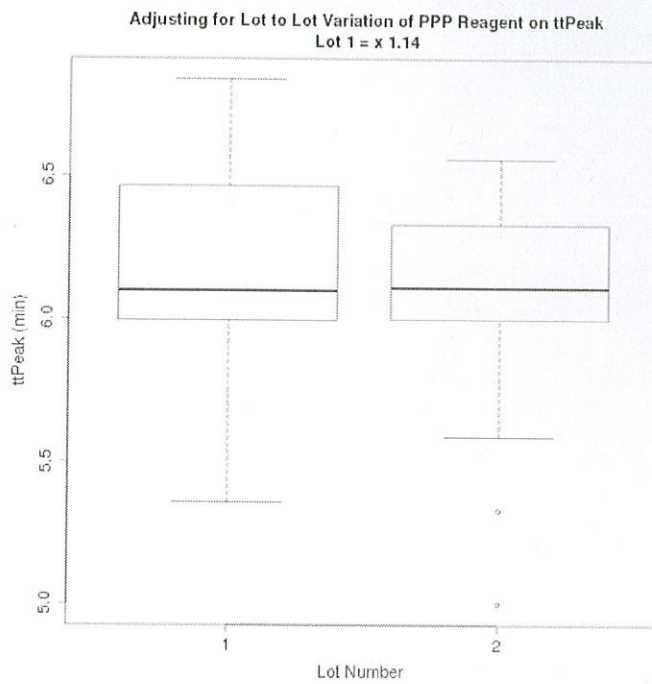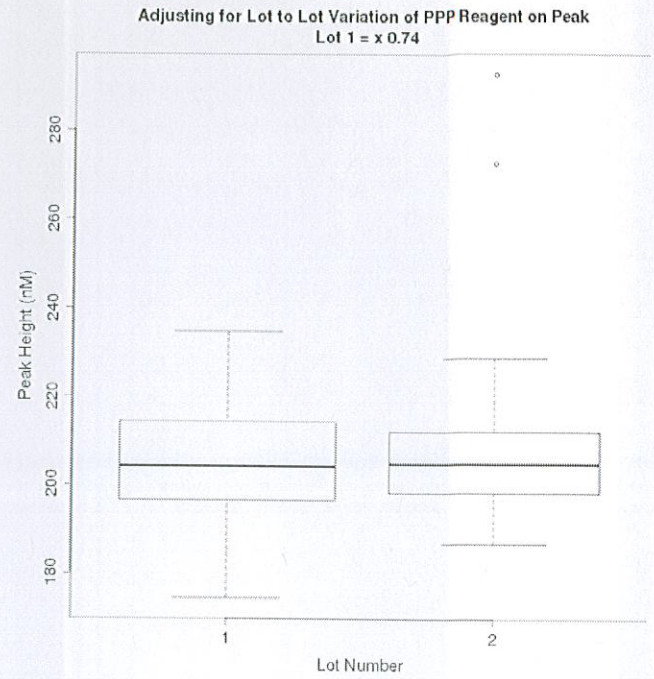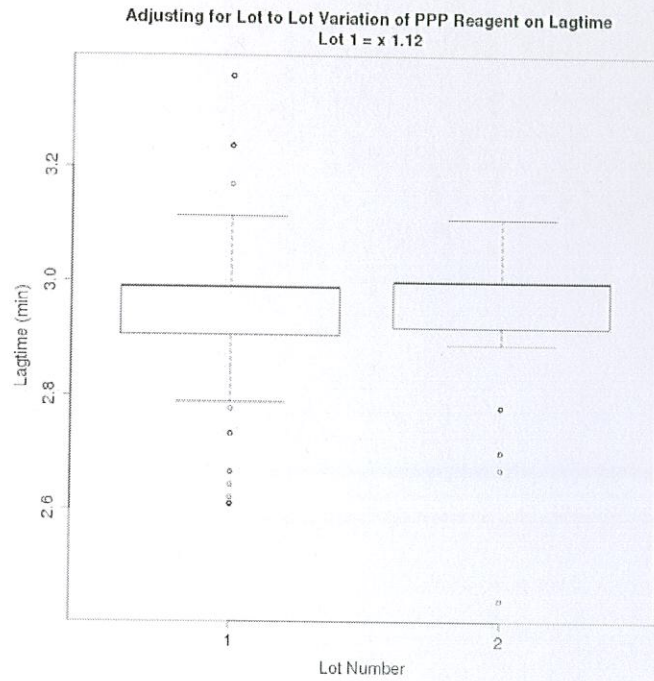Does not apply

Appendix:

Figure 1:

a)   Lot to lot variation of PPP reagents sold by Thrombinoscope BV accounted for during calculation of CAT parameters: Lag Time (min), Peak Height (nM) and ttPeak (min). The Graphs depicts the adjusted CAT results of Cryocheck reference plasma

- Lot-to-lot variation between lot #1 (original) and lot#2 (new)

b)   After adjustment:

Adjusting for Lot to Lot Variation of PPP Reagent on Lagtime
Lot 1 = x 1.12

Adjusting for Lot to Lot Variation of PPP Reagent on Peak
Lot 1 = x 0.74

Adjusting for Lot to Lot Variation of PPP Reagent on ttPeak
Lot 1 = x 1.14

Thrombin generation and procoagulant microparticle profiles after acute trauma: A prospective cohort study

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OBJECTIVE: The two sides of trauma-induced coagulopathy, the hypocoagulable and the hypercoagulable states, are poorly understood. To identify potential mechanisms for venous thromboembolism and bleeding after acute trauma, we estimated changes in circulating procoagulant microparticles (MPs) and thrombin activity during hospitalization for trauma.

METHODS: Whole blood was collected by venipuncture into 3.2% trisodium citrate at 0, 6, 12, 24, and 72 hours after injury and discharge. Platelet-poor plasma was harvested and stored at −80°C until analysis. Thrombin generation was determined using the calibrated automated thrombogram (CAT), reported as lag time (minutes), peak height (nM thrombin), and time to reach peak height (ttPeak, minutes). The concentration of total procoagulant MPs (number/μL) was measured by flow cytometry. Data are presented as median (interquartile range [IQR]).

RESULTS: Among 443 trauma patients (1,734 samples; Injury Severity Score [ISS], 13.0 [IQR, 6.0–22.0]; hospital length of stay, 4.0 days [IQR, 2.0–10.0]; age, 48 years [IQR, 28–65]; 70.7% male; 95% with blunt mechanism; mortality, 3.2%), no discernable patterns in thrombin generation or MP concentration were observed over time. The peak height and MPs were significantly different from healthy volunteers and were 337 nM (IQR, 285–395) and 400/μL plasma (IQR, 211–772), respectively. Extreme (defined as highest or lowest 5%) values reflecting a possible "hypercoagulable state" (lag time ≤ 1.98, peak height ≥ 486.2, ttPeak ≤ 3.61, and total procoagulant MP ≥ 2,278) were reached within 12 hours after acute trauma, while extreme values representing a possible "hypocoagulable state" (lag time ≥ 18.6, peak height ≤ 17.8, and ttPeak ≥ 29.45) were not reached until 1 day to 3 days.

CONCLUSION: Although there was no predictable pattern of coagulopathy observed in each patient after trauma, those who reached extreme values did so relatively early after injury. These findings should be taken into account when designing risk model tools involving coagulation laboratory parameters. (*J Trauma Acute Care Surg.* 2015;79: 726–731. Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.)

LEVEL OF EVIDENCE: Epidemiologic study, level III.

KEY WORDS: Trauma; thrombin; microparticle; prospective; cohort.

Trauma-induced coagulopathy (TIC) detected early after injury reflects injury severity and is prognostic for blood transfusion requirement and death.[1,2] Maintenance of hemostasis, often with blood products aimed at limiting hemorrhage, comes with the price of increased risk of venous thromboembolism (VTE).[3–5] The two sides of TIC, the hypocoagulable and the hypercoagulable states, are poorly understood. Existing therapies to treat TIC are based on limited understanding of their mechanisms. Hence, assays with enhanced sensitivity and specificity are needed to understand the basis for TIC. In a previous study, we noted that the concentration of plasma procoagulant microparticles (MPs) and peak thrombin generation in patients with blunt trauma correlated with injury severity, while the standard clotting assays (prothrombin time and activated partial thromboplastin time) were within the normal range.[6] Our long-term goal was to identify potential mechanisms for VTE and bleeding after acute trauma. In a prospective cohort study, we estimated serial changes in plasma procoagulant MP concentration and thrombin generation potential over time among patients hospitalized for acute trauma. We hypothesized that TIC occurs early after injury and it is quantifiable.

PATIENTS AND METHODS

In a prospective cohort study, all trauma patients transported to the Mayo Clinic Emergency Department (ED) by ambulance or air transport from February 2011 to June 2014 were considered for inclusion. Exclusion criteria were age less than 18 years, anticoagulation (e.g., heparin, warfarin) or antithrombotic therapy (excluding aspirin or nonsteroidal anti-inflammatory drugs), preexisting coagulopathy, more than 12 hours from time of injury, transfusion of blood products before blood sample collection, active cancer, sepsis, renal failure, burn injuries, or declined consent by the patient or legal guardian.

The time of injury was determined by the prehospital medical providers based on information at the injury scene. If the time of injury was unclear, the prehospital medical providers estimated the time and relayed this information to the emergency communication center. A trauma alert page was then sent to the hospital and laboratory staff as to the time of injury. We collected demographic and baseline clinical characteristics, including Injury Severity Score (ISS), patient age and sex, body mass index, hospital length of stay, all-cause mortality, and start and stop of anticoagulant-based thromboprophylaxis and other medications affecting coagulation. Transfusion therapy was mainly based not only on Mayo Clinic Trauma Center transfusion guidelines but also at the discretion of the medical provider. Blood samples also were collected for reference (control) analysis from 89 volunteers with no history of thrombosis (i.e., stroke, myocardial infarction, or VTE) or recent antithrombotic (thienopyridine; including aspirin or nonsteroidal anti-inflammatory drugs) or anticoagulant (heparin, low-molecular-weight heparin, warfarin) therapy, who were recruited by advertisement within the Mayo Clinic Employee Portal. This study was approved by the Mayo Clinic Institutional Review Board.

Sample Collection and Processing

Blood samples were collected at baseline; at 2, 6, and 12 hours; at 1 and 3 days after injury; and at hospital discharge. When patients were unable to provide consent at the time of the trauma, consent was obtained from the patient or legal guardian within 30 days of hospital discharge; samples were discarded when consent could not be obtained. A total of 18 mL of whole blood was collected by antecubital venipuncture or via existing indwelling catheters into 4.5 mL citrated Vacutainer tubes (0.105-M buffered sodium citrate, 3.2% Becton Dickinson, Plymouth, United Kingdom), processed to platelet free plasma by double centrifugation (3,000 G, 15 minutes) as recommended by the ISTH vascular biology SSC Collaborative Workshop,[7] and stored in multiple aliquots at −80°C until analysis. All samples were processed within 1 hour of collection.

CAT Analyses

Thrombin generation was measured with the CAT (Thrombinoscope BV, Maastricht, the Netherlands), using a Fluoroskan Ascent plate reader (390-nm excitation, 460-nm emission, Thermo Electron Corp, Vantaa, Finland), as previously described by Hemker et al.[8,9] and Owen et al.[10] Assays of trauma patient samples were performed in triplicate. Corn trypsin inhibitor (25 mg/mL) 25 μL (50 μg/mL, final concentration) was added to each plasma sample before CAT analyses. Thrombin generation was initiated using two different reagents: addition of 20 μL of either PPP (5-pM tissue factor and 4-μM phospholipid, Stago, United States) or PRP (1-pM tissue factor, Stago, US) reagent. Then, 80 μL of citrated platelet-poor plasma was added to each well of U-bottom 96-well microtiter plates (Nunc, Thermo Fischer Scientific, Rochester, NY) using a single channel pipette. After an incubation period (10 minutes at 37°C), 20 μL of warmed FLUCA reagent (Fluca kit, TS50, Thrombinoscope, BV), which contains the fluorogenic substrate and $CaCl_2$ was added to each well via an automated dispenser. Thrombin generation curves were recorded continuously for 90 minutes at a rate of three readings per minute. Separate wells

containing the thrombin calibrator, which corrects for inner filter effects and quenching variation among individual plasmas, were also measured in parallel.[9] A dedicated program, Thrombinoscope, was used to calculate thrombin activity over time. The parameters derived were lag time (LT), time to peak (ttPeak), and peak height (PH). We did not analyze samples collected after anticoagulant-based (mainly heparin) prophylaxis; we have found that prophylactic dose of heparin completely attenuates thrombin generation in CAT analyses. When unfractionated heparin was added, at varying concentrations from 0.1 U/mL to 0.4 U/mL, to platelet-poor plasma (Cryocheck), we noted a significant decrease in PH values (85% decrease) and prolongation of LT (200% increase) even at the lowest dose.

MP Analyses

The flow cytometric assay to measure plasma MPs, without ultracentrifugation, was adapted from the method of Ayers et al.[11] The MPs were analyzed by FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and using BD Diva Software version 6.0. Samples were run at high flow rate of 120 μL/min. To note, every morning, before patient sample analyses, filtered HEPES-buffered saline (HBS) buffers with and without antibodies were analyzed to be sure the reagents were free of contaminants. Fluorbrite beads of 0.2 μm to 2.0 μm in 1:10,000 dilution of HBS buffers were analyzed to ensure the instrument was optimized for gating on MPs, which were defined in this study as events less than 1 μm in diameter and positive for Annexin V (procoagulant) and cell-specific markers.

To establish procoagulant properties of the MP, a dual-labeling procedure was used. For each sample analysis, 100 μL of test plasma samples were diluted with 890 μL of filtered HBS (pH 7.4) and 10 μL of Hirudin (to achieve final concentration of 1 μM) was added to prevent clot formation. Then, all the samples were stained with 4-μL fluorochrome-labeled Annexin V–FITC (BD Pharmingen, 556420) monoclonal antibody, which binds to procoagulant phosphatidylserine (Annexin V) expressed on MP, and either 4 μL of R-phycoerythrin (PE)–labeled monoclonal antibody to CD42a, which binds to single chain integral membrane glycoprotein, GPIX, on platelet-derived MPs (BD Pharmingen, 558819 or 561853) or 4 μL of IgG PE (isotype control). Following 30-minute incubation of samples with the antibodies, 800 μL of filtered HBS-Ca was added as well as 100-μL Trucount beads (BD Biosciences). Each tube was run for a minimum of 300 seconds or until 1,500 events were collected in the Trucount bead gate. Trucount beads facilitated the accurate calculation of MP absolute numbers, using the following formula:

$$\text{Absolute count of cell population} = \frac{\text{Number of events in quadrant containing cell population}}{\text{Number of events in absolute count bead region}} \times \frac{\text{Total number of beads per test*}}{\text{Test volume}}$$

An MP gate on a flow cytometry plot of forward scatter versus side scatter was used to distinguish MP from small platelets, as previously published.[12,13] All buffers were filtered twice through Millex-GP 33-mm filters in preparation of daily sample analyses. All antibodies were also filtered twice through 0.2-μm membrane filters. Unfiltered buffers and antibodies contain interfering numbers of chemical MPs (data not shown). In addition, we used a commercially available reference plasma, Cryocheck (Precision Biologic, Dartmouth, NS), which was

TABLE 1. Demographic and Baseline Characteristics of 443 Trauma Patients

Age at trauma, median (IQR), y	48 (28–65)
Male sex, n (%)	313 (70.7)
Blunt mechanism, n (%)	423 (95.5)
ISS, median (IQR)	13.0 (6.0–22.0)
Hospital length of stay, median (IQR), d	4.0 (2.0–10.0)
Death, n (%)	14 (3.2)
International normalized ratio (admission), median (IQR)	1.0 (1.0–1.1)
Partial thromboplastin time (admission), median (IQR), s	26.0 (24.0–29.0)
Hemoglobin (admission), median (IQR), g/dL	13.7 (12.0–14.8)
Red blood cell, median (IQR) (first 24 h of arrival),* U	4 (2–7)
Plasma median (IQR) (first 24 h of arrival)*	5 (2–6)
Platelet median (IQR) (first 24 h of arrival),* U	2 (1–2)
Cryoprecipitate median (IQR) (first 24 h of arrival),* U	2 (1–2)
Tranexamic acid administration in prehospital or ED (%)	10 (2.3)
Bebulin (PCC) infusion in prehospital or ED (%)	4 (1)

*Median (IQR) among the patients who received each type of blood product within the first 24 hours of arrival (77 patients received red blood cells, 43 patients received plasma, 42 patients received platelets, and 14 patients received cryoprecipitate).

analyzed with every carousel of patient samples to ensure that our technique for MP analysis was consistent. The overall mean of AnnV pos MP counts in Cryocheck was calculated to be 3,046 ± 574 per μL plasma. Between two experienced research technologists, the range of coefficient of variation using this reference plasma has consistently been 15% to 23%. With the use of the compensation setup feature of DIVA software (V6, BD Biosciences), compensation was determined by running unstained and single-color positive control samples following the manufacturer's recommendations.

Statistical Analyses

Data analysis was performed using SAS version 9.3 (SAS Institute Inc, Cary, NC) and R version 3.0.2. Descriptive statistics are presented as median (interquartile range). A comparison of continuous variables between trauma patients and volunteers (controls) was performed using analysis of variance, the dependent variable being the ranked laboratory value and including age and sex in the model as additional independent variables. "Extreme" values were defined as the greatest or lowest 5% of values, regardless of patient or the time of sample acquisition relative

to the time of injury. For each laboratory parameter, the most "hypercoagulable" value within the first 24 hours for each patient was identified; this was defined as the lowest 5% LT and ttPeak, the greatest 5% PH and MP counts. The most "hypocoagulable" value was defined as values at the other end of extreme 5%. The α level was set at 0.05 for statistical significance.

RESULTS

From February 2011 through April 2014, we screened 2,106 patients and 1,418 met inclusion criteria. Of these eligible patients, 288 declined to participate, 332 patients did not return mail-in consents, and their study samples were discarded. Of the 798 enrolled, we randomly analyzed blood samples of 443 patients; to assess for existence of discernible pattern in thrombin generation and MP counts after injury. The number of patient samples analyzed at each time point was as follows: (1) 228 baseline, (2) 368 at 6 hours, (3) 391 at 12 hours, (4) 310 at 24 hours, (5) 210 on Day 3, and (6) 155 on the day of discharge. Demographic data are displayed in Table 1. Of the 443 patients sampled, 90 were transfused within the first 24 hours after arrival to our trauma center. The median among the patients who received each type of blood product within this time frame were as follows: packed red blood cells (RBCs) 4 (IQR, 2–7), frozen or thawed plasma 5 (IQR, 2–6), platelets 2 (IQR, 1–2), and cryoprecipitate 2 (IQR, 1–2).

Comparison of Laboratory Values Between Trauma Patients and Volunteers

The median patient and volunteer ages were 48 years (IQR, 28–65) and 40 years (IQR, 27–53) ($p = 0.002$), respectively; 71% and 49% of trauma patients and volunteers, respectively, were men ($p < 0.001$). For both PPP and PRP, thrombin PH and ttPeak were significantly greater and shorter, respectively, as compared with volunteers, while the LT did not differ significantly using PPP reagent. These samples from trauma patients also had significantly greater procoagulant MP levels in peripheral blood even after adjusting for age and sex (Table 2). We also observed a wide variance of thrombin generation and MP levels as compared with healthy volunteers (see Figures, Supplementary Digital Contents 1 and 2, http://links.lww.com/TA/A638).

TABLE 2. Comparison of Laboratory Values between Trauma Patients and Uninjured Screened Volunteers (Adjusted for Sex and Age)

Variable	Patient, Median (IQR)	Volunteer, Median (IQR)	p
5-pM TF/4-μM PS (PPP reagent) LT, min	2.67 (2.38–3.27)	2.67 (2.33–2.96)	0.320
PH, nM	337.6 (285.9–395.6)	320.6 (287.0–343.9)	0.0081
ttPeak, min	4.73 (4.19–5.56)	5.11 (4.61–5.67)	<0.0001
1-pM TF (PRP reagent) LT, min	8.34 (6.89–10.56)	9.00 (8.00–10.07)	<0.001
PH, nM	49.2 (33.9–71.4)	25.6 (19.8–34.1)	<0.0001
ttPeak, min	17.19 (14.55–20.22)	20.56 (18.56–22.78)	<0.0001
Total procoagulant MPs (number/μL plasma)	401 (212–772)	241 (146–530)	0.0015
Platelet-derived procoagulant MPs (number/μL plasma)*	19.4 (10.6–40.8)	31.6 (13.1–65.8)	0.0023

PS, phospholipid; TF, tissue factor.

Trend Over Time and Time To Reach Extreme Values

The plots of each individual patient's thrombin activity and MP counts over time did not exhibit a discernable pattern; each patient displayed a unique pattern after injury (see Figure, Supplementary Digital Content 3a–d, http://links.lww.com/TA/A638). Since no particular trends in the serial blood analyses of individual patients were observed, extreme (defined as highest or lowest 5%) values reflecting a possible "hypercoagulable state" (Tables 3–5) and "hypocoagulable state" were calculated (Tables 3 and 4). We observed that the hypercoagulable extreme values were reached within 12 hours after acute trauma, while hypocoagulable extreme values were not reached until 1 day to 3 days.

Comparisons of CAT and MP Values With Degrees of Injury Severity and Blood Transfusions

Among the cohort of 443 patients, 261 patients presented with ISS of less than 15, 96 patients presented with ISS of 15 to 24, and 86 patients had severe injury with ISS of 25 or greater. We observed that shortened LT, decreased PH, and ttPeak were found to be associated with greater injury severity (Table 6). However, there were no significant differences between these groups of patients with regard to MP counts. There were no overall significant differences in CAT and MP values between those who received any blood type transfusion and those who did not.

DISCUSSION

In this study, we have observed that endogenous thrombin generation and circulating procoagulant MP are significantly different in trauma patients when compared with volunteers. In particular, PH and ttPeak values were greater and lesser, respectively, in the trauma patients relative to the volunteers. Hence, patients exhibit acceleration of thrombin generation, indicating that the plasma coagulome is important in generating the hypercoagulable milieu needed for thrombotic complications. As injury severity increased, a shortened LT and ttPeak were observed, which are consistent with accelerated rate of thrombin generation.

In our cohort study, serial blood draws, relative to time of injury, were obtained from trauma patients until their discharge. In so doing, we did not observe any discernible pattern over

time for individual patient-level thrombin activity and MP counts. Curry et al.[14] recently showed, in their pilot study of 50 patients, an increase in peak thrombin generation immediately after trauma admission as compared with the values at the time of admission. However, the mean time from injury to admission was not specified. Similarly, Matijevic et al.[15] describe their findings of cellular MPs and thrombogram in patients enrolled in the Prospective Multicenter Major Trauma Transfusion (PROMMTT) study. They only collected one time blood sample at the time of admission for trauma, but the time at which these samples were collected relative to the time of injury is not specified. Obtaining blood relative to time of injury is important because it allows us to gain a better understanding of where, in the time continuum of injury response, a patient may reside. Regardless, this study supports the findings of these other investigators who have shown that thrombin generation and procoagulant MP counts are altered after trauma as compared with noninjured volunteers.[7,14,15]

Thrombin Generation Assay

CAT is a functional assay of global thrombin generation. It measures the rate of thrombin generation and inhibition in citrated plasma and has been used to quantify procoagulant activity in several diseases including VTE and coronary artery disease.[16–18] In our previous pilot study, we performed native thrombin generation assays, as initially described by Dunbar and Chandler,[19] in which no agonist such as tissue factor or phospholipid source (PCPS) was added. The role of CAT in clinical practice has yet to be defined largely because of a lack of official standardization of the assay with its associated large interlaboratory variability. However, Dargaud et al.[20] were able to reduce assay variability when assays were performed using

TABLE 4. Time to Extreme Values for CAT Data Using PRP* Reagent

Variable	n	Extreme 5% Value	Time to Extreme Value, d
Hypercoagulable			
LT, min	1,574	≤5.33	0.25 (0.07–0.47)
PH, nM	1,574	≥133.2	0.07 (0.05–0.26)
ttPeak, min	1,574	≤10.89	0.20 (0.06–0.45)
Hypocoagulable			
LT, min	1,574	≥18.56	2.98 (0.95–4.87)
PH, nM	1,574	≤17.8	1.86 (0.75–4.00)
ttPeak, min	1,574	≥29.45	2.95 (0.93–5.69)

*PRP, 1-pM TF.
TF, tissue factor.

TABLE 3. Time to Extreme Values for CAT Data Using PPP* Reagent

Variable	n	Extreme 5% Value	Time to Extreme Value, d
Hypercoagulable			
LT, min	1,575	≤1.98	0.27 (0.12–0.50)
PH, nM	1,575	≥486.2	0.50 (0.09–2.97)
ttPeak, min	1,575	≤3.61	0.32 (0.24–0.59)
Hypocoagulable			
LT, min	1,575	≥5.00	2.95 (1.03–7.27)
PH, nM	1,575	≤194.6	0.97 (0.49–3.08)
ttPeak, min	1,575	≥8.00	2.95 (0.93–6.68)

*PPP, 5-pM TF/4-µM PS.
PS, phospholipid; TF, tissue factor.

TABLE 5. Time to Extreme Values for MPs

Variable	n	Extreme 5% Value	Time to Extreme Value, d
CD42+, procoagulant MPs (/µL plasma)*	1,734	≥126	0.55 (0.25–4.06)
Total procoagulant MPs (/µL plasma)	1,734	≥2,278	0.25 (0.05–1.98)

*CD42+, procoagulant MPs, Annexin V+ MPs derived from platelets only.

TABLE 6. Comparisons of CAT and MP Values With Degrees of Injury Severity

Variable	ISS < 15 (n = 261)	ISS, 15–24 (n = 96)	ISS ≥ 25 (n = 86)	p
5-pM TF/4-µM PS (PPP reagent) LT, min	2.79 (2.38–3.33)	2.67 (2.33–3.11)	2.67 (2.20–3.36)	<0.0001
PH, nM	342.6 (293.2–396.8)	347.4 (290.2–409.9)	319.4 (267.6–376.2)	<0.0001
ttPeak, min	4.94 (4.33–5.78)	4.56 (4.06–5.14)	4.52 (3.92–5.30)	<0.0001
1-pM TF (PRP reagent) LT, min	8.67 (7.11–11.00)	7.86 (6.67–10.22)	8.22 (6.64–10.21)	<0.0001
PH, nM	46.9 (33.2–68.1)	49.8 (34.0–73.4)	52.4 (35.9–77.5)	0.0071
ttPeak, min	17.89 (15.33–21.00)	16.70 (14.05–19.36)	15.97 (13.56–18.84)	<0.0001
Total procoagulant MPs (/µL plasma)	405.6 (211.5–786.9)	424.3 (237.9–770.7)	372.7 (197.8–758.3)	0.370
CD42⁺, procoagulant MPs (/µL plasma)*	20.6 (11.1–42.1)	18.5 (9.9–36.1)	19.0 (9.1–42.9)	0.0520

*CD42$^+$, procoagulant MPs, platelet-derived, procoagulant MPs.
PS, phospholipid; TF, tissue factor.

identical equipment, standardized reagents, and carefully selected reference plasma for normalization of results. We have previously demonstrated that CAT assays can be performed with reproducible results when commercially available reference plasma is used with every patient sample runs.[21] In addition, the presence of lot-to-lot variability of PPP and PRP reagents have to be taken into account if CAT data are to be used to make clinical correlations. In brief, we plan to perform experiments that hopefully will begin to dissect the procoagulant and anticoagulant activities accounting for the observed net thrombin generation response after trauma. The investigators of Trans-Agency Research Consortium for Trauma-Induced Coagulopathy (TACTIC) study 1-UM-1-HL120877-2 have begun the performance of comprehensive analyses of coagulation and inflammation profiles in trauma patients. Hopefully, this multicenter multidisciplinary team of physicians and scientists can enhance our understanding of the mechanisms for the development of coagulopathy in trauma patients after injury.

MP Analyses by Flow Cytometry

MPs are found in healthy individuals. In normal plasma, MP derived from platelets are the most common (>80%), followed by MPs derived from endothelial cells (<10%) and leukocytes (<10%).[22] Thrombogenic potential of MPs that express surface phosphatidylserine (Annexin V+) are procoagulant in vitro.[13,22,23] Thus, endogenous MPs expressing membrane phosphatidylserine may not contribute intrinsically to thrombosis but may drive thrombosis in an environment where procoagulant enzymes are generated, such as would be expected in peripheral blood of patients after major trauma.[7] In this study, MP analyses were limited to Annexin V–binding (procoagulant) MPs, some of which are derived from platelets. Further evaluation needs to be performed to quantify the number and subtypes of MPs present in our bank of stored samples. Curry et al. quantified subtypes of MPs in platelet-poor plasma without ultracentrifugation. We adopted their technique as data variability became significantly less when ultracentrifugation step was omitted. Curry et al. found significantly greater red blood cell, procoagulant (Annexin V+) MPs, and decrease in endothelial cell–derived MPs. As compared with their 41 survivors, the 9 nonsurvivors had significant decrease in platelet-derived procoagulant MPs. As performed by Curry et al., we have used the recommendations within the ISTH SSC guidance for standardization of MP analysis.[24] Similar to CAT assays, an obstacle to translating MP analysis methods into the clinical arena is the lack of standardization that has been uniformly accepted by the research community. Namely, there is a lack of consensus with regard to MP analyses performed using identical equipment, standardized reagents, and carefully selected reference plasma for normalization of results. This is an area of research in dynamic flux, and it remains to be determined if characterization of procoagulant MPs could find practical applications in the clinical arena.

Limitations

Our study has several limitations. First, our definitions of hypercoagulable or hypocoagulable states were arbitrary. We chose the greatest or least 5% of thrombin generation variables to define hypercoagulable and hypocoagulable states, respectively. With regard to total procoagulant MPs, we defined plasma samples with the greatest number (in the top 5%) to be hypercoagulable. Second, the patients enrolled in this study were not consecutive admissions because our overall consent rate was approximately 60%. Lastly, we did not stratify our findings based on fluid administration and blood transfusions, which may affect our findings secondary to hemodilution or alteration of coagulation secondary to blood product administration.

CONCLUSION

In this prospective cohort study, we observed an increase in thrombin generation by CAT and procoagulant MPs after injury. Although there was no predictable pattern of coagulopathy observed in each patient after acute trauma, patients who reached extreme values did so relatively early after injury. These findings should inform the design of risk model tools involving coagulation laboratory parameters. Challenges remain regarding the lack of standardization and lack of reference controls to allow reproducibility of data among different centers. Hence, it remains to be determined if thrombin assays by CAT and characterization of procoagulant MPs could find practical applications as diagnostic indicators of postinjury TIC.

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